

Nine microsatellite DNA primers for *Hippophae rhamnoides* ssp. *sinensis* (Elaeagnaceae)

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Abstract *Hippophae rhamnoides* ssp. *sinensis* occurs mainly in the arid regions of northwest China. The wood stands of this subspecies play an important role in maintaining the local ecosystems in these regions. In addition, the genetic characteristics are essential to understand the historical range changes of this subspecies and its morphological differentiation with other subspecies. In this study, we developed nine microsatellite loci for this subspecies for the first time. We used the combining biotin capture method to enrich AG/CT/AC/GT/CG/GTG/CCA microsatellites. Twenty-six microsatellites were isolated from the enriching library and nine of them were found to be polymorphic through screening 12 distantly distributed individuals. The number of alleles per locus ranged from three to twelve and expected heterozygosity from 0.2659 to 0.4767, respectively. We further performed cross-priming tests in another subspecies and two congeneric species. These firstly isolated loci will provide a useful tool to investigate the genetic structure of this subspecies and its morphological differentiation from the other subspecies.

Keywords *Hippophae rhamnoides* ssp. *sinensis* · Microsatellite markers · Population genetics

Hippophae is a small genus of Elaeagnaceae with about five species (Lian et al. 1998). All species can fix the nitro in the air and therefore are important candidates in forest restoration (Tian et al. 2004). In addition, the fossil pollens of this genus were well preserved in the lake sediments or deep soils and are usually used to indicate the past climatic oscillations (Tang and Shen 1996). However, little is known regarding the genetic diversity within and between populations of each species. This information is undoubtedly important to understand the range shifts of each of them in the history. In addition, one species among them, *Hippophae rhamnoides*, is highly variable and divided into three to nine subspecies according to the different authors (Bartish et al. 2002). The genetic variations are also important to elucidate such a complex morphological divergence. In this study, we isolated nine microsatellite markers for *H. rhamnoides* ssp. *sinensis*.

The total genomic DNA was extracted from the silica gel dried leaves using DNeasyTM Tissue Kit (Qiagen). The microsatellite regions were isolated following Zhang et al. (2007). About 500 ng genomic DNA was digested into approximately 500 bp fragments with a restriction enzyme *Rsa*I (NEB) and *Xmn*I (NEB), then ligated to SuperSNX24 double-stranded adaptors (mixture of equal volumes of equal molar amounts of SuperSNX24-F: 5'-GTTTAA GGCCTAGCTAGCAGAATC-3' + SuperSNX24 + 4P-R: 5'-GATTCTGCTAG-CTAGGCCTTAAACAAAA-3'). For enrichment, the ligation products were hybridized with an oligonucleotide combination of 5'-biotinylated probes, (AG)₁₅, (CT)₁₅, (AC)₁₅, (GT)₁₅, (CG)₁₅, (GTG)₁₂, and (CCA)₁₂. The hybridization in the 50 µl solution (2× SSC, 1 µmol/l probe and 10 µl ligation products) was as follows: an initial 5 min at 95°C, then a rapid cooling to 70°C followed by 0.2°C incremental decreases every 5 s for 99 cycles, and maintenance at 50°C for 10 min; then

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decreases of 0.5°C every 5 s for 20 cycles, and finally rapid cooling to 15°C. The DNA hybridized to the probe was captured by streptavidin-coated magnetic beads at 37°C for 1 h and then washed by the solution I (2× SSC, 0.1% SDS) and solution II (1× SSC, 0.1% SDS). The captured DNA was recovered by polymerase chain reactions (PCR) with SuperSNX-F (5'-GTTTAAGGCCTAGCTAGCAGAATC-3') and PCR product was purified with TIANquick Midi Purification Kit (TIANGEN). These fragments enriched with microsatellite loci were cloned using pMD 18-T vector (Takara) and transformed into the *E. coli* competent cell (JM109, Takara). Positive colonies were amplified using BcaBEST™ Sequencing Primers RV-M and BcaBEST™ Sequencing Primers M13-47. Only those PCR products, sized between 300 and 600 bp were sequenced on ABI3130 Automated DNA Analysis System using the BigDye Ready Reaction Terminator Kit following the manufacture's protocols. The sequences containing motifs repeating more than 5 times were regarded as microsatellites. A total of 26 sequences were identified among the sequenced 200 sequences and primer pairs for amplification of the microsatellite regions were designed using the Primer 5.0 (Clarke and Gorley 2001).

In order to check polymorphisms of the identified microsatellite loci, 12 individuals from distantly distributed populations were selected for test. The PCR reactions were performed in 25 µl reaction mixtures with 10–40 ng

template DNA, containing 19 µl of sterile double distilled water; 2.5 µl of 10× Taq polymerase reaction buffer; 1 µl each of each of the primers; 1 unit Taq DNA polymerase. The amplifications used an initial denaturation of 5 min at 94°C, and then followed by 38 cycles of 94°C for 40 s, annealing for 40 s at 48–51°C, 72°C for 45 s plus a final extension of 72°C for 10 min. PCR products were initially checked for PCR amplification on 2.0% agarose gels. The successful PCR products were further resolved on 6.5% polyacrylamide denaturing gel using a 50 bp DNA ladder (Takara) as the reference and visualized by silver staining. We calculated observed and expected heterozygosity (H_O and H_E) using GENEPOP version 3.4 (<http://wbiomed.curtin.edu.au/genepop/>) (Raymond and Rousset 1995).

Among tested 26 SSRs, 17 were monomorphic and nine loci revealed multi-banding patterns. The number and range of the amplified alleles were determined across all sampled individuals. The size of the amplified fragments ranged from 70 to 218 bp (Table 1). These loci yielded 3–12 alleles per locus and most of the alleles were sequenced and verified to be the target sequences. All subspecies and species of *Hippophae* are diploid (Bartish et al. 2002). The observed heterozygosity and expected heterozygosity therefore were calculated to range from 0.1397 to 0.2997 and from 0.2659 to 0.4767, respectively. For each locus, the expected heterozygosity was always significantly bigger than the observed heterozygosity

Table 1 Characteristics of nine polymorphic microsatellite loci for *Hippophae rhamnoides* ssp. *sinensis*

Locus	Primers sequence (5'–3')	Repeat	Ta (°C)	N	Size range (bp)	No. of alleles	H_O	H_E	GenBank Accession No.
Hr01	F: GGGATCGCCAAATAAACT R: TCGGTTGGTGCTTCGTTT	(GA) ₁₁	51	12	180–218	12	0.1397	0.2659	EU089748
Hr02	F: CGTCTTTCAATCGACACTTATTC R: GCTAGAAGAGTCTTTTCGTTGCA	(AG) ₉	48	12	165–205	6	0.2997	0.4767	EU089749
Hr03	F: TCAATCCAAACTTGTTTCGCC R: TATCGATTTCTCCCAACTG	(AG) ₁₀	50	12	81–111	11	0.1587	0.2939	EU089750
Hr04	F: ATCACATGGCAATACATTTTTTC R: TCAAATCACCCAAAACCTGAACAC	(CA) ₈	50	12	101–131	4	0.2321	0.3939	EU089751
Hr05	F: CTTGCCGCCGTGAGCTCTAG R: GCAATCATCGTCTCTTCTTCTCCC	(GTG) ₅	50	12	201–216	3	0.2997	0.4767	EU089752
Hr06	F: CAACAAAATACAATTCGAAAC R: AATAGGAGACACAGAGGCTTC	(CA) ₉	50	12	70–90	12	0.1591	0.2958	EU089753
Hr07	F: AGAATCACAAGGCTTCACCAC R: TAGTCCCTTTGAGGTTGTAG	(CCA) ₅	50	12	129–148	5	0.2690	0.4354	EU089754
Hr08	F: TAGCTAGATAGAATCTTATGTTG R: CTCGGACGCACTTTGTCTCTTTG	(TC) ₂₆	50	12	180–192	3	0.2997	0.4767	EU089755
Hr09	F: GCGGGAATTCGATTAAGG R: TGAGTAGAGGGCTACCAAC	(TG) ₅	50	12	182–196	4	0.2321	0.3939	EU089756

The number of repeats is based on the sequence of the cloned alleles

Ta, annealing temperature; N, number of individuals genotyped; H_O , observed heterozygosity; H_E , expected heterozygosity

Table 2 Cross-priming tests in another subspecies and three congeneric species

Species	Locus								
	Hr01	Hr02	Hr03	Hr04	Hr05	Hr06	Hr07	Hr08	Hr09
<i>H. rhamnoides</i> ssp. <i>turkestanica</i>	+	+	+	+	+	+	–	+	+
<i>H. gyantsensis</i>	–	–	+	+	+	+	+	+	+
<i>H. neurocarpa</i>	+	–	+	+	+	+	–	+	+
<i>H. goniocarpa</i>	–	+	+	+	+	+	–	+	+

At least two individuals of each taxon were screened. Successful amplification indicated as +, unsuccessful amplification as –

($P < 0.05$). No significant genotypic disequilibrium was detected for any pair of loci.

We further performed cross-priming tests in another subspecies and the other three congeneric species. Only eight loci were successfully amplified in *H. rhamnoides* ssp. *turkestanica* and seven in the other three species: *H. gyantsensis*, *H. neurocarpa* and *H. goniocarpa* (Table 2). The highly variable SSR loci isolated in this study are the first set of molecular markers designed specifically for *H. rhamnoides* ssp. *sinensis*. These markers will provide a powerful means of assessing the genetic diversity overall its natural distributions, which may further elucidate its range changes in response to the historical climatic oscillations and morphological divergence with other subspecies and species.

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